

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
8 May 2003 (08.05.2003)

PCT

(10) International Publication Number  
**WO 03/038085 A1**

(51) International Patent Classification<sup>7</sup>: **C12N 9/88, C07D 309/00**

(21) International Application Number: **PCT/GB02/04951**

(22) International Filing Date: **30 October 2002 (30.10.2002)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:  
0126165.0 31 October 2001 (31.10.2001) **GB**  
60/343,447 21 December 2001 (21.12.2001) **US**

(71) Applicant (for all designated States except US):  
**DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).**

(72) Inventors; and

(75) Inventors/Applicants (for US only): **MORGAN, Andrew, John [GB/DK]; Fredensvej 1, DK-2950 Vedbæk (DK). REFDAHL, Charlotte [DK/DK]; Holmeås 51, DK-2670 Greve (DK). YU, Shukun [SE/SE]; G. Hejdemansgatan 29, S-212 40 Malmö (SE).**

(74) Agents: **ALCOCK, David et al.; D Young & Co, 21 New Fetter Lane, London EC4A 1DA (GB).**

(81) Designated States (national): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.**

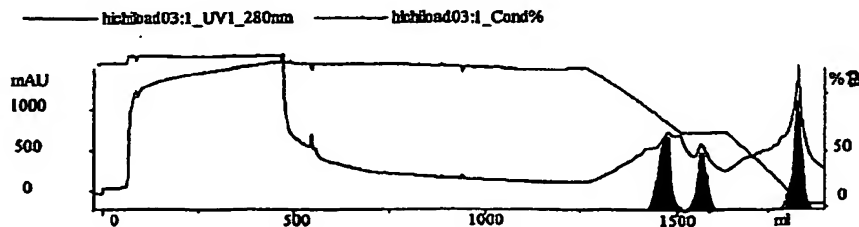
(84) Designated States (regional): **ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).**

**Published:**

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **1,5-ANHYDRO-D-FRUCTOSE DEHYDRATASE**



(57) Abstract: The present invention relates to the purification and characterisation of 1,5-anhydro-D-fructose dehydratase.

WO 03/038085 A1

BEST AVAILABLE COPY

## 1,5-ANHYDRO-D-FRUCTOSE DEHYDRATASE

### FIELD OF THE INVENTION

- 5 The present invention relates to the purification and characterisation of 1,5-anhydro-D-fructose dehydratase.

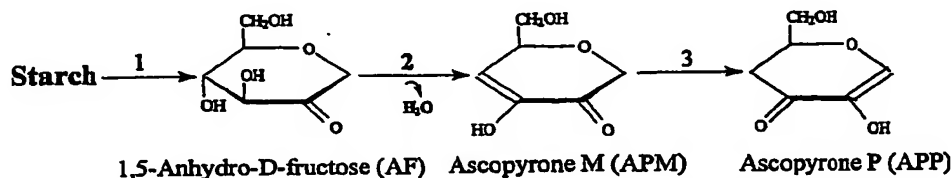
### TECHNICAL BACKGROUND AND PRIOR ART

- 10 It is known in the art that Ascopyrone P (APP) is a good antioxidant, antibrowning agent, and antimicrobial [WO 00/56838 filed 16/3/00, claiming priority from GB9906457.8, filed 19/3/99; Wo 02/26060 filed 27/9/01, claiming priority from GB023686.9 and GB0023687.7, both filed 27/9/00]. APP was first prepared from amylopectin, amylose and cellulose by pyrolysis, but the yield of APP was less than 3.0
- 15 % [Shafizadeh, F., Furneaux R.H., Stevenson, T.T., and Cochran, T.G., Carbohydr. Res. 67(1978): 433-447]. APP was later isolated from fungi of the order *Pezizales*, such as *Anthracoëbia melaloma*, *Plicaria anthracina*, *P. leiocarpa*, and *Peziza petersi* [M.-A. Baute, G. Deffieux, J. Vercauteren, R. Baute, and A. Badoc., Phytochemistry, 33 (1993): 41-45].
- 20 It was presumed by Baute *et al* [1993, *ibid*] that APP is formed enzymatically from maltodextrins or glycogen via 1,5-anhydro-D-fructose (AF). It was also presumed by that the conversion of AF to an unstable ketoenol (ascopyrone M, APM) is catalysed by a so-called conventional dehydratase which can be inhibited by EDTA [M.-A. Baute,
- 25 G. Deffieux, R. Baute, A. Badoc, J. Vercauteren, J.-M. Léger, and A. Neveu, Phytochemistry, 30 (1991): 1419-1423]. However, none of the enzymes involved were isolated or characterised by Baute *et al*.
- It was in 1997 and 1999 that fungal  $\alpha$ -1,4-glucan lyase (EC 4.2.2.13) which converts
- 30 starch-type substrates to AF was first purified, characterised and cloned [Yu. S.; Christensen TMIE, Kragh KM, Bojsen K, Marcussen J, Biochim Biophys Acta 1339: 311-320 (1997); Bojsen K.; Yu, S.; Marcussen J, Plant Mol Biol. 40: 445-454 (1999)].

2

Further work has indicated that the formation of APP from AF requires the action of two enzymes in tandem, i.e., AFDH and APS. AFDH converts AF to an intermediate with a maximum absorbance at 263nm (most likely APM, see Scheme 1). The conversion of this intermediate APM to APP then requires the action of APS.

5



Scheme 1

10 Scheme 1 illustrates the proposed formation of ascopyrone P (APP) from starch-type substrates (starch, dextrans, maltosaccharides, and glycogen etc.). The reactions catalysed are: 1,  $\alpha$ -1,4-glucan lyase (EC 4.2.2.13); 2, 1,5-anhydro-D-fructose dehydratase (AFDH, EDTA-insensitive), and 3, Ascopyrone P synthase (APS) (enolone or ketoenol isomerase, enolone or ketoenol tautomerase).

15

The AFDH disclosed herein has not been previously been reported in the fungus *Anthracobia melaloma*.

## SUMMARY OF THE INVENTION

20

In a broad aspect the invention relates to the purification and characterisation of 1,5-anhydro-D-fructose dehydratase.

25

Aspects of the present invention are presented in the claims and in the following commentary.

For ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

**DETAILED DISCLOSURE OF INVENTION**

The present invention relates to 1,5-Anhydro-D-fructose dehydratase in isolated or purified form.

5

**PREFERRED ASPECTS**

Preferably, the 1,5-anhydro-D-fructose dehydratase of the invention is obtainable from *Anthracobia Melaloma*.

10

In a preferred embodiment of the invention, the 1,5-Anhydro-D-fructose dehydratase has a temperature optimum of from about 34 to 50 °C.

15

In a more preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase has a temperature optimum of about 38 °C.

In a preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase has an optimal pH range of from about 5.9 to about 7.0.

20

Even more preferably, the 1,5-Anhydro-D-fructose dehydratase has an optimal pH range of from about 6.0 to about 7.0.

More preferably still, the 1,5-Anhydro-D-fructose dehydratase of the invention has an optimal pH of about 6.7.

25

In another preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase of the invention is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.

30

In yet another preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase of the invention has the following characteristics:

- (i) a temperature optimum of about 34 to about 50 °C;
- (ii) an optimal pH range of from about 5.9 to about 7.0; and

- (iii) is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.

In another preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase of the invention has the following characteristics:

- (i) a temperature optimum of about 34 to about 50 °C;  
(ii) an optimal pH of about 6.7; and  
(iii) is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.

10

In another preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase of the invention has the following characteristics:

- (i) a temperature optimum of about 38 °C;  
(ii) an optimal pH range of from about 5.9 to about 7.0; and  
15 (iii) is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.

In another preferred embodiment, the 1,5-Anhydro-D-fructose dehydratase of the invention has the following characteristics:

- 20 (i) a temperature optimum of about 38 °C;  
(ii) an optimal pH of about 6.7; and  
(iii) is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.

25 Preferably, the 1,5-Anhydro-D-fructose dehydratase of the invention is sensitive to one or more of  $\text{ZnCl}_2$ , EDTA and DTT.

Even more preferably, the 1,5-Anhydro-D-fructose dehydratase of the invention is inhibited by one or more of  $\text{ZnCl}_2$ , EDTA and DTT.

30

Preferably, the 5-Anhydro-D-fructose dehydratase of the invention is sensitive to one or more of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Na}^+$  ions.

Even more preferably, the 5-Anhydro-D-fructose dehydratase exhibits enhanced activity in the presence of one or more of  $Mg^{2+}$ ,  $Ca^{2+}$  and  $Na^{+}$  ions.

Another aspect of the invention relates to a process for preparing ascopyrone P using  
5 the 1,5-anhydro-D-fructose dehydratase of the invention.

Preferably, the process further comprises the use of ascopyrone P synthase.

Even more preferably, the process comprises contacting ascopyrone P synthase and the  
10 1,5-anhydro-D-fructose dehydratase of the invention with 1,5-anhydro-D-fructose.

In a preferred embodiment, the process comprises contacting ascopyrone P synthase and the 1,5-anhydro-D-fructose dehydratase of the invention with hydrated 1,5-anhydro-D-fructose. Surprisingly, improved activity is observed when hydrated 1,5-anhydro-D-fructose is used as the substrate.  
15

In a particularly preferred embodiment, the process further comprises the use of  $\alpha$ -1,4-glucan lyase.

20 In one preferred embodiment, the process comprises contacting  $\alpha$ -1,4-glucan lyase, ascopyrone P synthase and the 1,5-anhydro-D-fructose dehydratase of the invention with a starch-type substrate.

As used herein, the term "starch-type substrate" includes, for example, glycogen, or an  
25 intermediate compound resulting from the hydrolysis of starch by amylase enzymes, such as a maltodextrin. Examples of starch-type substrates include starch, amylopectin, amylose and dextrin.

Preferably, the starch-type substrate is selected from glycogen or a maltodextrin.

30

In another preferred embodiment comprises the steps of:

(i) contacting  $\alpha$ -1,4-glucan lyase with a starch-type substrate;

6

(ii) contacting the product from step (i) with ascopyrone P synthase and 1,5-anhydro-D-fructose dehydratase of the invention.

Another aspect relates to a process for preparing ascopyrone M using the 1,5-anhydro-D-fructose dehydratase of the invention.

Preferably, said process comprises contacting the 1,5-anhydro-D-fructose dehydratase of the invention with 1,5-anhydro-D-fructose.

Yet another aspect relates to a process for preparing cortalcerone using the 1,5-anhydro-D-fructose dehydratase of the invention.

Preferably, said process comprises contacting the 1,5-anhydro-D-fructose dehydratase of the invention with glucosone.

## ADVANTAGES

The present invention relates to the purification and characterisation of 1,5-anhydro-D-fructose dehydratase. To date, this enzyme has been neither isolated nor purified.

Furthermore, the enzyme of the present invention may be used in the production of APP. APP is itself useful as, *inter alia*, an anti-microbial material.

## ASSAY

The following assay may be used to characterise and identify actual and putative amino acid sequences according to the present invention.

## ISOLATED

In one aspect, preferably the enzyme is in an isolated form. The term "isolated" means that the enzyme is not in its natural environment (i.e. as found in nature). Typically the term "isolated" means that the enzyme is at least substantially free from at least one

other component with which the enzyme is naturally associated in nature and as found in nature. Here, the enzyme may be separated from at least one other component with which it is naturally associated.

## 5 PURIFIED

In one aspect, preferably the enzyme is in a purified form. The term "purified" also means that the enzyme is not in its natural environment (i.e. as found in nature). Typically the term "purified" means that the enzyme is at least substantially separated  
10 from at least one other enzyme with which the enzyme is naturally associated in nature and as found in nature.

## NUCLEOTIDE SEQUENCE

15 The present invention encompasses nucleotide sequences encoding enzymes having the specific properties as defined herein. The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-  
20 stranded or single-stranded whether representing the sense or antisense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence of the present invention.

25

In a preferred embodiment, the nucleotide sequence *per se* of the present invention does not cover the native nucleotide sequence according to the present invention in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred  
30 embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated,



which promoter is also in its native environment. However, the amino acid sequence of the present invention can be isolated and/or purified post expression of a nucleotide sequence in its native organism. Preferably, however, the amino acid sequence of the present invention may be expressed by a nucleotide sequence in its native organism but  
5 wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

Typically, the nucleotide sequence of the present invention is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative  
10 embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

## 15 PREPARATION OF THE NUCLEOTIDE SEQUENCE

A nucleotide sequence encoding either an enzyme which has the specific properties as defined herein or an enzyme which is suitable for modification may be identified and/or isolated and/or purified from any cell or organism producing said enzyme. Various  
20 methods are well known within the art for the identification and/or isolation and/or purification of nucleotide sequences. By way of example, PCR amplification techniques to prepare more of a sequence may be used once a suitable sequence has been identified and/or isolated and/or purified.

By way of further example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the enzyme. If the amino acid sequence of the enzyme is known, labelled oligonucleotide probes may be synthesised and used to identify enzyme-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide  
30 probe containing sequences homologous to another known enzyme gene could be used to identify enzyme-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, enzyme-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for enzyme (i.e. maltose),  
5 thereby allowing clones expressing the enzyme to be identified.

In a yet further alternative, the nucleotide sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beaucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869,  
10 or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed  
15 synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in  
20 Saiki R K *et al* (Science (1988) 239, pp 487-491).

## AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of enzymes having the  
25 specific properties as defined herein.

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino  
30 acid sequence" is synonymous with the term "enzyme".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be

made synthetically or it may be prepared by use of recombinant DNA techniques.

The enzyme of the present invention may be used in conjunction with other enzymes. Thus the present invention also covers a combination of enzymes wherein the combination comprises the enzyme of the present invention and another enzyme, which  
5 may be another enzyme according to the present invention. This aspect is discussed in a later section.

Preferably the enzyme is not a native enzyme. In this regard, the term "native enzyme"  
10 means an entire enzyme that is in its native environment and when it has been expressed by its native nucleotide sequence.

#### VARIANTS/HOMOLOGUES/DERIVATIVES

15 The present invention also encompasses the use of variants, homologues and derivatives of any amino acid sequence of an enzyme of the present invention or of any nucleotide sequence encoding such an enzyme. Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

20

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although  
25 homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, an homologous sequence is taken to include a nucleotide  
30 sequence which may be at least 40, 50, 60, 70, 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to a nucleotide sequence encoding an enzyme of the present invention (the subject sequence). Typically, the homologues will comprise the

same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

5

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

10

% Homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

15

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed.

20

Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

25

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap

30

penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

5

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software than  
10 can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al* 1999 Short Protocols in Molecular Biology, 4<sup>th</sup> Ed – Chapter 18), FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). However, for some  
15 applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

20 Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST  
25 suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

30

Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS<sup>TM</sup> (Hitachi Software), based on an algorithm, analogous to

**THIS PAGE BLANK (USPTO)**

CLUSTAL (Higgins DG & Sharp PM (1988), Gene 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such

**THIS PAGE BLANK (USPTO)**



as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine  
5 (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids include; alpha\* and alpha-disubstituted\* amino acids, N-alkyl amino acids\*, lactic acid\*, halide derivatives of  
10 natural amino acids such as trifluorotyrosine\*, p-Cl-phenylalanine\*, p-Br-phenylalanine\*, p-I-phenylalanine\*, L-allyl-glycine\*,  $\beta$ -alanine\*, L- $\alpha$ -amino butyric acid\*, L- $\gamma$ -amino butyric acid\*, L- $\alpha$ -amino isobutyric acid\*, L- $\epsilon$ -amino caproic acid<sup>#</sup>, 7-amino heptanoic acid\*, L-methionine sulfone<sup>#\*</sup>, L-norleucine\*, L-norvaline\*, p-nitro-L-phenylalanine\*, L-hydroxyproline<sup>#</sup>, L-thioprolin\*, methyl derivatives of  
15 phenylalanine (Phe) such as 4-methyl-Phe\*, pentamethyl-Phe\*, L-Phe (4-amino)<sup>#</sup>, L-Tyr (methyl)\*, L-Phe (4-isopropyl)\*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)\*, L-diaminopropionic acid<sup>#</sup> and L-Phe (4-benzyl)\*. The notation \* has been utilised for the purpose of the discussion above (relating to homologous or non-homologous substitution), to indicate the hydrophobic nature of the derivative whereas  
20 # has been utilised to indicate the hydrophilic nature of the derivative, #\* indicates amphipathic characteristics.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as  
25 methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or  $\beta$ -alanine residues. A further form of variation involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the  $\alpha$ -carbon substituent group is on the residue's nitrogen atom  
30 rather than the  $\alpha$ -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ *et al.*, PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Suitable fragments will be at least 5, e.g. 10, 12, 15 or 20 amino acids in length. They may also be less than 100, 75 or 50 amino acids in length. They may contain one or more (e.g. 5, 10, 15 or 20) substitutions, deletions or insertions, including conserved substitutions.

5

The nucleotide sequences for use in the present invention may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the  
10 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences of the present invention.

15 The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences presented herein, or any homologue, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

20

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different  
25 populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA  
30 libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues

and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

10

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

20

The present invention also encompasses polynucleotides which have undergone molecular evolution via random processes, selection mutagenesis or in vitro recombination. As a non-limiting example, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either in vivo or in vitro, and to subsequently screen for improved functionality of the encoded polypeptide by various means. In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wildtype or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide. The production of new preferred variants can be achieved by various methods well established in the art, for example the Error Threshold Mutagenesis (WO 92/18645), oligonucleotide mediated random mutagenesis

30

(US 5,723,323), DNA shuffling (US 5,605,793), exo-mediated gene assembly WO 00/58517. The application of these and similar random directed molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting

sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

### BIOLOGICALLY ACTIVE

Preferably, the variant sequences etc. are at least as biologically active as the sequences presented herein.

As used herein "biologically active" refers to a sequence having a similar structural function (but not necessarily to the same degree), and/or similar regulatory function (but not necessarily to the same degree), and/or similar biochemical function (but not necessarily to the same degree) of the naturally occurring sequence.

### ISOZYMES

The polypeptide of the present invention may exist in the form of one or more different isozymes. As used herein, the term "isozyme" encompasses variants of the polypeptide that catalyse the same reaction, but differ from each other in properties such as substrate affinity and maximum rates of enzyme-substrate reaction. Owing to differences in amino acid sequence, isozymes can be distinguished by techniques such as electrophoresis or isoelectric focusing. Different tissues often have different isoenzymes. The sequence differences generally confer different enzyme kinetic parameters that can sometimes be interpreted as fine tuning to the specific requirements of the cell types in which a particular isoenzyme is found.

## ISOFORMS

The present invention also encompasses different isoforms of the 1,5-anhydro-D-fructose dehydratase described herein. The term "isoform" refers to a protein having  
5 the same function (namely 1,5-anhydro-D-fructose dehydratase activity), which has a similar or identical amino acid sequence, but which is the product of a different gene.

## HYBRIDISATION

10 The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary thereto.

The term "hybridisation" as used herein shall include "the process by which a strand of  
15 nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

The present invention also encompasses the use of nucleotide sequences that are  
20 capable of hybridising to the sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof.

The term "variant" also encompasses sequences that are complementary to sequences  
25 that are capable of hybridising to the nucleotide sequences presented herein.

Preferably, the term "variant" encompasses sequences that are complementary to  
sequences that are capable of hybridising under stringent conditions (e.g. 50°C and  
0.2xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na<sub>3</sub>citrate pH 7.0}) to the nucleotide  
sequences presented herein.

30 More preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C

and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na<sub>3</sub>citrate pH 7.0}) to the nucleotide sequences presented herein.

5 The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of the present invention  
10 (including complementary sequences of those presented herein).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency.

15

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

20 In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

#### **SITE-DIRECTED MUTAGENESIS**

25

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to mutate the sequence in order to prepare an enzyme of the present invention.

30 Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649), wherein a single-stranded gap of DNA, the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. The synthetic nucleotide, bearing the desired mutation, is then annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase.

US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the above mentioned Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151). This method involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesised DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

By way of example, Sierks *et al* (Protein Eng (1989) 2, 621-625 and Protein Eng (1990) 3, 193-198) describes site-directed mutagenesis in *Aspergillus* glucoamylase.

## RECOMBINANT

In one aspect of the present invention the sequence is a recombinant sequence – i.e. a sequence that has been prepared using recombinant DNA techniques.

## SYNTHETIC

In one aspect of the present invention the sequence is a synthetic sequence – i.e. a



sequence that has been prepared by *in vitro* chemical or enzymatic synthesis. It includes but is not limited to sequences made with optimal codon usage for host organisms, such as the methylotrophic yeasts *Pichia* and *Hansenula*.

## 5 EXPRESSION OF ENZYMES

The nucleotide sequence for use in the present invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in enzyme form, in and/or from a compatible host cell. Both  
10 homologous and heterologous expression is contemplated.

As used with reference to the present invention, the terms "expression", "expresses", "expressed" and "expressible" are synonymous with the respective terms "transcription", "transcribes", "transcribed" and "transcribable".

15

For homologous expression, preferably the gene of interest or nucleotide sequence of interest is not in its naturally occurring genetic context. In the case where the gene of interest or nucleotide sequence of interest is in its naturally occurring genetic context, preferably expression is driven by means other than or in addition to its naturally  
20 occurring expression mechanism; for example, by overexpressing the gene of interest by genetic intervention.

Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters  
25 and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The enzyme produced by a host recombinant cell by expression of the nucleotide  
30 sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a

particular prokaryotic or eukaryotic cell membrane.

## EXPRESSION VECTOR

5 The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated in the genome of a suitable host organism. The term "incorporated" preferably covers stable incorporation into the genome.

10

The host organism can be the same or different to the gene of interest source organism, giving rise to homologous and heterologous expression respectively.

15 Preferably, the vector of the present invention comprises a construct according to the present invention. Alternatively expressed, preferably the nucleotide sequence of the present invention is present in a vector and wherein the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

20

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide of the present invention. Thus, in a further aspect the invention provides a process for preparing polypeptides for subsequent use according to the present invention which comprises cultivating a host cell transformed or transfected with an expression vector under conditions to provide 25 for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

30 The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The choice of vector will often depend on the host cell into which it is to be introduced.

The vectors of the present invention may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Suitable selection markers may be the *dal* genes from *B. subtilis* or *B. licheniformis*,  
5 or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternative selection markers may be the *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, or a marker giving rise to hygromycin resistance. Examples of other fungal selection markers are the genes for ATP synthetase, subunit 9 (*oliC*), orotidine-5'-phosphate-decarboxylase (*pvrA*),  
10 phleomycin and benomyl resistance (*benA*). Examples of non-fungal selection markers are the bacterial G418 resistance gene (this may also be used in yeast, but not in filamentous fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uidA* gene, coding for  $\beta$ -glucuronidase (GUS). Further suitable selection markers include the *dal* genes from *B. subtilis* or *B. licheniformis*.  
15 Alternatively, the selection may be accomplished by co-transformation (as described in WO 91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

20

Thus, nucleotide sequences for use according to the present invention can be incorporated into a recombinant vector (typically a replicable vector), for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method  
25 of making nucleotide sequences of the present invention by introducing a nucleotide sequence of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

30

The procedures used to ligate a DNA construct of the invention encoding an enzyme which has the specific properties as defined herein, and the regulatory sequences, and to

insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (for instance see Sambrook *et al* Molecular Cloning: A laboratory Manual, 2<sup>nd</sup> Ed. (1989)).

- 5 The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The expression vector typically includes the components of a cloning vector, such as, for  
10 example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences encoding a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression vector may comprise a  
15 sequence coding for an amino acid sequence capable of targeting the amino acid sequence to a host cell organelle such as a peroxisome or to a particular host cell compartment. In the present context, the term 'expression signal' includes any of the above control sequences, repressor or activator sequences. For expression under the direction of control sequences, the nucleotide sequence is operably linked to the control sequences in proper  
20 manner with respect to expression.

## REGULATORY SEQUENCES

In some applications, the nucleotide sequence for use in the present invention is  
25 operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

30

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory

sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

- 5 The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

10

- Enhanced expression of the nucleotide sequence encoding the enzyme of the present invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the enzyme of the present invention. In eukaryotes, polyadenylation sequences may be operably connected to the nucleotide sequence encoding the enzyme.
- 15

- Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.
- 20

- Aside from the promoter native to the gene encoding the nucleotide sequence of the present invention, other promoters may be used to direct expression of the polypeptide of the present invention. The promoter may be selected for its efficiency in directing the expression of the nucleotide sequence of the present invention in the desired expression host.
- 25

- In another embodiment, a constitutive promoter may be selected to direct the expression of the desired nucleotide sequence of the present invention. Such an expression construct may provide additional advantages since it circumvents the need to culture the expression hosts on a medium containing an inducing substrate.
- 30

Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial host include the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens*  $\alpha$ -amylase gene (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes and a promoter derived from a *Lactococcus* sp.-derived promoter including the P170 promoter. When the nucleotide sequence is expressed in a bacterial species such as *E. coli*, a suitable promoter can be selected, for example, from a bacteriophage promoter including a T7 promoter and a phage lambda promoter.

For transcription in a fungal species, examples of useful promoters are those derived from the genes encoding the, *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xlnA*), phytase, ATP-synthetase, subunit 9 (*oliC*), triose phosphate isomerase (*tpi*), alcohol dehydrogenase (*AdhA*),  $\alpha$ -amylase (*amy*), amyloglucosidase (AG - from the *glaA* gene), acetamidase (*amdS*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters. Other examples of useful promoters for transcription in a fungal host are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber *et al* (1982) J. Mol. Appl. Genet. 1, p419-434), *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Examples of suitable promoters for the expression in a yeast species include but are not

limited to the Gal 1 and Gal 10 promoters of *Saccharomyces cerevisiae* and the *Pichia pastoris* AOX1 or AOX2 promoters.

Hybrid promoters may also be used to improve inducible regulation of the expression  
5 construct.

The promoter can additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoter may even contain other sequences to affect (such  
10 as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the Sh1-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the  
15 TMV 5' signal sequence (see Sleat 1987 Gene 217, 217-225 and Dawson 1993 Plant Mol. Biol. 23: 97).

## CONSTRUCTS

20 The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence for use according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present  
25 invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

30 The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a bacterium, preferably of the genus *Bacillus*, such as *Bacillus subtilis*, or plants into which it has been transferred. Various markers exist which

may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

- 5 For some applications, preferably the construct of the present invention comprises at least the nucleotide sequence of the present invention operably linked to a promoter.

### HOST CELLS

- 10 The term "host cell" - in relation to the present invention includes any cell that comprises either the nucleotide sequence or an expression vector as described above and which is used in the recombinant production of an enzyme having the specific properties as defined herein. The nucleotide of interest may be homologous or heterologous to the host cell.

15

- Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence that expresses the enzyme of the present invention. Preferably said nucleotide sequence is carried in a vector for the replication and expression of the nucleotide sequence. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial),  
20 fungal, yeast or plant cells.

- Examples of suitable bacterial host organisms are gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*,  
25 *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus megaterium* and *Bacillus thuringiensis*, *Streptomyces* species such as *Streptomyces murinus*, lactic acid bacterial species including *Lactococcus* spp. such as *Lactococcus lactis*, *Lactobacillus* spp. including *Lactobacillus reuteri*, *Leuconostoc* spp., *Pediococcus* spp. and  
30 *Streptococcus* spp. Alternatively, strains of a gram-negative bacterial species belonging to *Enterobacteriaceae* including *E. coli*, or to *Pseudomonadaceae* can be selected as the host organism.



The gram negative bacterium *E. coli* is widely used as a host for heterologous gene expression. However, large amounts of heterologous protein tend to accumulate inside the cell. Subsequent purification of the desired protein from the bulk of *E. coli* intracellular proteins can sometimes be difficult.

5

In contrast to *E. coli*, Gram positive bacteria from the genus *Bacillus*, such as *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium*, *B. thuringiensis*, *Streptomyces lividans* or *S. murinus*, may be very  
10 suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria that may be suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

Depending on the nature of the nucleotide sequence encoding the enzyme of the present  
15 invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different  
20 fungal host organism should be selected.

Typical fungal expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger* var. *tubigenis*, *Aspergillus niger* var. *awamori*, *Aspergillus aculeatis*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Bacillus subtilis*,  
25 *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

Suitable filamentous fungus may be for example a strain belonging to a species of *Aspergillus*, such as *Aspergillus oryzae* or *Aspergillus niger*, or a strain of *Fusarium*  
30 *oxysporium*, *Fusarium graminearum* (in the perfect state named *Gibberella zeae*, previously *Sphaeria zeae*, synonym with *Gibberella roseum* and *Gibberella roseum* f. sp. *Cerealis*), or *Fusarium sulphureum* (in the perfect state named *Gibberella*

*puricaris*, synonym with *Fusarium trichothercioides*, *Fusarium bactridioides*, *Fusarium sambucium*, *Fusarium roseum* and *Fusarium roseum* var. *graminearum*), *Fusarium cerealis* (synonym with *Fusarium crookwellense*) or *Fusarium venenatum*.

- 5 Suitable yeast organisms may be selected from the species of *Kluyveromyces*, *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*, or *Hansenula* (disclosed in UK Patent Application No. 9927801.2).

10 The use of suitable host cells - such as yeast, fungal and plant host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

- 15 The host cell may be a protease deficient or protease minus strain. This may for example be the protease deficient strain *Aspergillus oryzae* JaL 125 having the alkaline protease gene named "alp" deleted. This strain is described in WO 97/35956.

## ORGANISM

20

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence according to the present invention when present in  
25 the organism.

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

30 The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the enzyme according to the present invention and/or the products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence according to the present invention within the

organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

5 The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the enzyme  
10 according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the products thereof. For example the transgenic organism can also comprise the nucleotide sequence coding for the enzyme of the present invention under the control of a  
15 heterologous promoter.

#### TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism.  
20 Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in  
25 Molecular Biology (1995), John Wiley & Sons, Inc. If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

Filamentous fungi cells may be transformed by a process involving protoplast  
30 formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

Another host organism can be a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

#### TRANSFORMED FUNGUS

A host organism may be a fungus - such as a mould. Examples of suitable such hosts include any member belonging to the genera *Thermomyces*, *Acremonium*, *Aspergillus*, *Penicillium*, *Mucor*, *Neurospora*, *Trichoderma* and the like - such as *Thermomyces lanuginosus*, *Acremonium chrysogenum*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, *Penicillium chrysogenum*, *Mucor javanicus*, *Neurospora crassa*, *Trichoderma viridae* and the like.

In one embodiment, the host organism may be a filamentous fungus.

For almost a century, filamentous fungi have been widely used in many types of industry for the production of organic compounds and enzymes. For example, traditional Japanese koji and soy fermentations have used *Aspergillus sp.* Also, in this century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons why filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, for example

enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous expression according to the present invention.

5

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting the nucleotide sequence according to the present invention into a construct designed for expression in filamentous fungi.

10 Several types of constructs used for heterologous expression have been developed. These constructs preferably contain one or more of: a signal sequence which directs the amino acid sequence to be secreted, typically being of fungal origin, and a terminator (typically being active in fungi) which ends the expression system.

15 Another type of expression system has been developed in fungi where the nucleotide sequence according to the present invention can be fused to a smaller or a larger part of a fungal gene encoding a stable protein. This can stabilise the amino acid sequence. In such a system a cleavage site, recognised by a specific protease, can be introduced between the fungal protein and the amino acid sequence, so the produced fusion protein  
20 can be cleaved at this position by the specific protease thus liberating the amino acid sequence. By way of example, one can introduce a site which is recognised by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo* resulting in production of the expressed product and not a larger fusion protein.

25 Heterologous expression in *Aspergillus* has been reported for several genes coding for bacterial, fungal, vertebrate and plant proteins. The proteins can be deposited intracellularly if the nucleotide sequence according to the present invention is not fused to a signal sequence. Such proteins will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins. If the nucleotide  
30 sequence according to the present invention is equipped with a signal sequence the protein will accumulate extracellularly.

With regard to product stability and host strain modifications, some heterologous proteins are not very stable when they are secreted into the culture fluid of fungi. Most fungi produce several extracellular proteases which degrade heterologous proteins. To avoid this problem special fungal strains with reduced protease production have been used as  
5 host for heterologous production.

Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N.*  
10 *crassa* is found, for example in Davis and de Serres, Methods Enzymol (1971) 17A:79-143. Standard procedures are generally used for the maintenance of strains and the preparation of conidia. Mycelia are typically grown in liquid cultures for about 14 hours (25°C), as described in Lambowitz *et al.*, J Cell Biol (1979) 82:17-31. Host strains can generally be grown in either Vogel's or Fries minimal medium  
15 supplemented with the appropriate nutrient(s), such as, for example, any one or more of: his, arg, phe, tyr, trp, p-aminobenzoic acid, and inositol.

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707 which states that once a construct has been obtained, it can be introduced either in  
20 linear form or in plasmid form, e.g., in a pUC-based or other vector, into a selected filamentous fungal host using a technique such as DNA-mediated transformation, electroporation, particle gun bombardment, protoplast fusion and the like. In addition, Ballance 1991 (*ibid*) states that transformation protocols for preparing transformed fungi are based on preparation of protoplasts and introduction of DNA into the protoplasts using  
25 PEG and Ca<sup>2+</sup> ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers.

To allow for selection of the resulting transformants, the transformation typically also involves a selectable gene marker which is introduced with the expression cassette,  
30 either on the same vector or by co-transformation, into a host strain in which the gene marker is selectable. Various marker/host systems are available, including the pyrG, argB and niaD genes for use with auxotrophic strains of *Aspergillus nidulans*; pyrG and

argB genes for *Aspergillus oryzae* auxotrophs; pyrG, trpC and niaD genes for *Penicillium chrysogenum* auxotrophs; and the argB gene for *Trichoderma reesei* auxotrophs. Dominant selectable markers including amdS, oliC, hyg and phleo are also now available for use with such filamentous fungi as *A. niger*, *A. oryzae*, *A. ficum*,  
5 *P. chrysogenum*, *Cephalosporium acremonium*, *Cochliobolus heterostrophus*, *Glomerella cingulata*, *Fulvia fulva* and *Leptosphaeria maculans* (for a review see Ward in Modern Microbial Genetics, 1991, Wiley-Liss, Inc., at pages 455-495). A commonly used transformation marker is the amdS gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole nitrogen source.

10

For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous. Among the markers used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance.

15

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

A transgenic *Aspergillus* according to the present invention can also be prepared by  
20 following the teachings of Rambosek, J. and Leach, J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In: Martinelli S.D., Kinghorn J.R. (Editors) *Aspergillus: 50 years on*. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance, D.J. 1991  
25 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In: Leong, S.A., Berka R.M. (Editors) Molecular Industrial Mycology. Systems and Applications for Filamentous Fungi. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R. (Editors) *Aspergillus: 50 years on*. Progress in industrial microbiology vol  
30 29. Elsevier Amsterdam 1994. pp. 641-666).

## TRANSFORMED YEAST

In another embodiment the transgenic organism can be a yeast.

- 5 In this regard, yeast have also been widely used as a vehicle for heterologous gene expression.

By way of example, the species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes  
10 in *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

- 15 For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of  
20 knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a  
25 vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating  
30 plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by



inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs may contain a promoter active in yeast, such as a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence  
5 of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be  
10 prepared by following the teachings of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

The transformed yeast cells may be selected using various selective markers. Among the  
15 markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

## TRANSFORMED PLANTS/PLANT CELLS

20

A preferred host organism suitable for the present invention is a plant.

In this respect, the basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the  
25 inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found  
30 in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Even though the promoter of the present invention is not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare transgenic plants according to the present invention. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Thus, in one aspect, the present invention relates to a vector system which carries a nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An *et al.* (1980), *Binary Vectors, Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An *et al.* (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non-limiting example of such a Ti plasmid is pGV3850.

The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a

T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

- 5 As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an  
10 *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants; many vector systems exist which are based on these plasmids or derivatives thereof.

- In the construction of a transgenic plant the nucleotide sequence or construct of the  
15 present invention may be first constructed in a micro-organism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful micro-organism is *E. coli*, but other micro-organisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli* it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g.  
20 *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain; e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

- 25 As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, the pUC series, the M13 mp series, pACYC 184 etc.

- 30 In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the

transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA  
5 sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the desired promoter or construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of  
10 further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The  
15 Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasterdam, 1985, Chapter V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An *et al.*, EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. *et al.* (1980), *Tissue Culture*  
20 *Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

25

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the promoter and/or the GOI, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown  
30 on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance

with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Other techniques for transforming plants include ballistic transformation, the silicon whisker carbide technique (see Frame BR, Drayton PR, Bagnaall SV, Lewnau CJ, Bullock WP, Wilson HM, Dunwell JM, Thompson JA & Wang K (1994) Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation, The Plant Journal 6: 941-948) and viral transformation techniques (e.g. see Meyer P, Heidmann I & Niedenhof I (1992) The use of cassava mosaic virus as a vector system for plants, Gene 110: 213-217). Teachings on ballistic transformation are presented in following section.

Further teachings on plant transformation may be found in EP-A-0449375.

## **20 BALLISTIC TRANSFORMATION OF PLANTS AND PLANT TISSUE**

As indicated, techniques for producing transgenic plants are well known in the art. Typically, either whole plants, cells or protoplasts may be transformed with a suitable nucleic acid construct encoding a zinc finger molecule or target DNA (see above for examples of nucleic acid constructs). There are many methods for introducing transforming DNA constructs into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods include *Agrobacterium* infection (see, among others, Turpen *et al.*, 1993, J. Virol. Methods, 42: 227-239) or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles. Acceleration methods are generally preferred and include, for example, microprojectile bombardment.

Originally developed to produce stable transformants of plant species which were recalcitrant to transformation by *Agrobacterium tumefaciens*, ballistic transformation of plant tissue, which introduces DNA into cells on the surface of metal particles, has found utility in testing the performance of genetic constructs during transient expression. In this way, gene expression can be studied in transiently transformed cells, without stable integration of the gene in interest, and thereby without time-consuming generation of stable transformants.

In more detail, the ballistic transformation technique (otherwise known as the particle bombardment technique) was first described by Klein *et al.* [1987], Sanford *et al.* [1987] and Klein *et al.* [1988] and has become widespread due to easy handling and the lack of pre-treatment of the cells or tissue in interest.

The principle of the particle bombardment technique is direct delivery of DNA-coated micro-projectiles into intact plant cells by a driving force (e.g. electrical discharge or compressed air). The micro-projectiles penetrate the cell wall and membrane, with only minor damage, and the transformed cells then express the promoter constructs.

One particle bombardment technique that can be performed uses the Particle Inflow Gun (PIG), which was developed and described by Finer *et al.* [1992] and Vain *et al.* [1993]. The PIG accelerates the micro-projectiles in a stream of flowing helium, through a partial vacuum, into the plant cells.

One of advantages of the PIG is that the acceleration of the micro-projectiles can be controlled by a timer-relay solenoid and by regulation the provided helium pressure. The use of pressurised helium as a driving force has the advantage of being inert, leaves no residues and gives reproducible acceleration. The vacuum reduces the drag on the particles and lessens tissue damage by dispersion of the helium gas prior to impact [Finer *et al.* 1992].

In some cases, the effectiveness and ease of the PIG system makes it a good choice for the generation of transient transformed guar tissue, which were tested for transient

expression of promoter/reporter gene fusions.

A typical protocol for producing transgenic plants (in particular monocotyledons), taken from U.S. Patent No. 5, 874,265, is described below:

5

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

10

A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming both dicotyledons and monocotyledons, is that neither the isolation of protoplasts nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the tungsten-DNA particles so that they are not delivered to the recipient cells in large aggregates. It is believed that without a screen intervening between the projectile apparatus and the cells to be bombarded, the projectiles aggregate and may be too large for attaining a high frequency of transformation. This may be due to damage inflicted on the recipient cells by projectiles that are too large.

15

20

25

30

For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more clusters of cells transiently expressing a marker gene ("foci") on the bombarded filter. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 2 to 3.

After effecting delivery of exogenous DNA to recipient cells by any of the methods discussed above, a preferred step is to identify the transformed cells for further culturing and plant regeneration. This step may include assaying cultures directly for a screenable trait or by exposing the bombarded cultures to a selective agent or agents.

5

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage, incubating the cells at, e.g., 18°C and greater than 180  $\mu\text{E m}^{-2} \text{ s}^{-1}$ , and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

10

An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

15

To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g. for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/l bialaphos. While ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not believed to be particularly crucial, and can comprise any solid, porous, inert support.

20

Cells that survive the exposure to the selective agent may be cultured in media that supports regeneration of plants. Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot development will signal the time to transfer to another media.

25

Regeneration typically requires a progression of media whose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential

30



developmental stages from the transformed callus to the more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO<sub>2</sub>, and 250  $\mu\text{E m}^{-2} \text{ s}^{-1}$  of light. Plants are preferably matured either in a growth chamber or greenhouse.

- 5   Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue culture vessels. An illustrative embodiment of such a vessel is a petri dish. Regenerating plants are preferably grown at about 19°C to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

10

Genomic DNA may be isolated from callus cell lines and plants to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art such as PCR and/or Southern blotting.

- 15   Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

20

## **CULTURING AND PRODUCTION**

- Host cells transformed with the nucleotide sequence may be cultured under conditions conducive to the production of the encoded enzyme and which facilitate recovery of the  
25   enzyme from the cells and/or culture medium.

- The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in questions and obtaining expression of the enzyme. Suitable media are available from commercial suppliers or may be prepared according to  
30   published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The protein produced by a recombinant cell may be displayed on the surface of the cell. If desired, and as will be understood by those of skill in the art, expression vectors containing coding sequences can be designed with signal sequences which direct secretion of the coding sequences through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ *et al* (1993) DNA Cell Biol 12:441-53).

The enzyme may be secreted from the host cells and may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

## SECRETION

Often, it is desirable for the enzyme to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces*, *Kluyveromyces* and *Hansenula*) or the  $\alpha$ -amylase gene (*Bacillus*).

## DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay

(ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the POI may be used or a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton R *et al* (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE *et al* (1983, J Exp Med 15 8:121 1).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labelled hybridization or PCR probes for detecting the amino acid sequence include oligolabelling, nick translation, end-labelling or PCR amplification using a labelled nucleotide. Alternatively, the NOI, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesise RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labelled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-4,277,437; US-A-4,275,149 and US-A-4,366,241. Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

Additional methods to quantitate the expression of the amino acid sequence include radiolabeling (Melby PC *et al* 1993 J Immunol Methods 159:235-44) or biotinylating (Duplaa C *et al* 1993 Anal Biochem 229:36) nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. Quantitation of multiple samples may be speeded up by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

Although the presence/absence of marker gene expression suggests that the nucleotide sequence is also present, its presence and expression should be confirmed. For example, if the nucleotide sequence is inserted within a marker gene sequence, recombinant cells containing nucleotide sequences can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a nucleotide sequence under the control of the promoter of the present invention or an alternative promoter (preferably the same promoter of the present invention). Expression of the marker gene in response to induction or selection usually indicates expression of the amino acid sequence as well.

10

Alternatively, host cells which contain the nucleotide sequence may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

15

## FUSION PROTEINS

The amino acid sequence of the present invention may be produced as a fusion protein, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

25

The fusion protein may comprise an antigen or an antigenic determinant fused to the substance of the present invention. In this embodiment, the fusion protein may be a non-naturally occurring fusion protein comprising a substance which may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The antigen or antigenic determinant may be attached to either the amino or carboxy terminus of the substance.

30

In another embodiment of the invention, the amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

### ADDITIONAL POIs

The sequences of the present invention may be used in conjunction with one or more additional proteins of interest (POIs) or nucleotide sequences of interest (NOIs).

Non-limiting examples of POIs include: proteins or enzymes involved in starch metabolism, proteins or enzymes involved in glycogen metabolism, acetyl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carboxypeptidases, catalases, cellulases, chitinases, chymosin, cutinase, deoxyribonucleases, epimerases, esterases,  $\alpha$ -galactosidases,  $\beta$ -galactosidases,  $\alpha$ -glucanases, glucan lysases, endo- $\beta$ -glucanases, glucoamylases, glucose oxidases,  $\alpha$ -glucosidases,  $\beta$ -glucosidases, glucuronidases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, rhamnogalacturonases, ribonucleases, thaumatin, transferases, transport proteins, transglutaminases, xylanases, hexose oxidase (D-hexose: O<sub>2</sub>-oxidoreductase, EC 1.1.3.5) or combinations thereof. The NOI may even be an antisense sequence for any of those sequences.

The POI may even be a fusion protein, for example to aid in extraction and purification.

Examples of fusion protein partners include the maltose binding protein, glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion components.

The POI may even be fused to a secretion sequence. Examples of secretion leader sequences are those originating from the amyloglucosidase gene, the  $\alpha$ -factor gene, the  $\alpha$ -amylase gene, the lipase A gene, the xylanase A gene.

- 5 Other sequences can also facilitate secretion or increase the yield of secreted POI. Such sequences could code for chaperone proteins as for example the product of *Aspergillus niger cyp B* gene described in UK patent application 9821198.0.

- 10 The NOI may be engineered in order to alter their activity for a number of reasons, including but not limited to, alterations which modify the processing and/or expression of the expression product thereof. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns or to change codon preference. By way of further example, the NOI may also be modified to optimise expression in a particular  
15 host cell. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites.

- The NOI may include within it synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include  
20 methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the NOI may be modified by any method available in the art. Such modifications may be carried out in to enhance the *in vivo* activity or life span of the NOI.

25

The NOI may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule.

30

## ANTIBODIES

One aspect of the present invention relates to amino acid sequences that are immunologically reactive with the amino acid sequences of the invention.

5

Antibodies may be produced by standard techniques, such as by immunisation with the substance of the invention or by using a phage display library.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, fragments produced by a Fab expression library, as well as mimetics thereof. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and F(ab')<sub>2</sub> fragments, as well as single chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. Furthermore, the antibodies and fragments thereof may be humanised antibodies. Neutralising antibodies, i.e., those which inhibit biological activity of the substance polypeptides, are especially preferred for diagnostics and therapeutics.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with the sequence of the present invention (or a sequence comprising an immunological epitope thereof). Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed if purified the substance polypeptide is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

30

Serum from the immunised animal is collected and treated according to known

procedures. If serum containing polyclonal antibodies to the sequence of the present invention (or a sequence comprising an immunological epitope thereof) contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against the sequence of the present invention (or a sequence comprising an immunological epitope thereof) can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

Monoclonal antibodies to the sequence of the present invention (or a sequence comprising an immunological epitope thereof) may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 *Nature* 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) *Immunol Today* 4:72; Cote *et al* (1983) *Proc Natl Acad Sci* 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984) *Proc Natl Acad Sci* 81:6851-6855; Neuberger *et al* (1984) *Nature* 312:604-608; Takeda *et al* (1985) *Nature* 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.



Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

5

Antibody fragments which contain specific binding sites for the substance may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-1281).

10

## LARGE SCALE APPLICATION

15

In one preferred embodiment of the present invention, the amino acid sequence is used for large scale applications.

20

Preferably the amino acid sequence is produced in a quantity of from 1g per litre to about 2g per litre of the total cell culture volume after cultivation of the host organism.

25

Preferably the amino acid sequence is produced in a quantity of from 100mg per litre to about 900mg per litre of the total cell culture volume after cultivation of the host organism.

30

Preferably the amino acid sequence is produced in a quantity of from 250mg per litre to about 500mg per litre of the total cell culture volume after cultivation of the host organism.

## SUMMARY

The invention is further illustrated in the following non-limiting examples with

reference to the following figures wherein:

Figure 1 shows the separation of AFDH, APS1 and APS2 by hydrophobic interaction chromatography on a HiLoad Phenyl Sepharose 16/10 HP column (Pharmacia). The solid line is absorbance at 280 nm (Y-axis right), the broken line is % of Buffer B (Y-axis left). The elution volume is indicated in the X-axis. The activity peaks are shaded. The first activity peak is AFDH, the second is APS1 and the third is APS2.

Figure 2 shows further purification of AFDH by ion exchange chromatography on a 6 ml resource Q column. The solid line is the absorbance at 280 nm (y-axis right), the broken line is % of buffer B2 (y-axis left). The elution volume is indicated in the X-axis. The AFDH activity peak is indicated as the shaded area.

Figure 3 shows further purification of AFDH on Mono Q HR 5/5 anion exchange column. The AFDH was eluted in an elution volume of 93 to 96 ml.

Figure 4 shows chromatography of AFDH by gel filtration chromatography on Superdex 200. AFDH showed an elution volume of 12 ml.

Figure 5 shows the purity examination of the finally purified AFDH (lane 1 to 3 from left) obtained by the gel filtration purification step. The analysis was SDS-PAGE followed by Coomassie blue staining. The protein markers in kDa from Novex are from above: 200 (myosin); 116.3 (beta-galactosidase); 97.4(phosphorylase b); 66.3(BSA); 55.4(glutamic dehydrogenase); 36.5(lactate dehydrogenase); 31.0 (carbonic anhydrase), 21.5 (trypsin inhibitor), 14.4 (lysozyme).

## EXAMPLES

### 1. Purification and characterisation of AFDH from the fungus *Anthracobia melaloma*

5

Anhydrofructose dehydratase (AFDH) was purified from *Anthracobia melaloma* with a purification factor of 409 fold achieved in 5 purification steps. AFDH showed a molecular mass of 98.5 kDa on SDS gel electrophoresis and 228 kDa by gel filtration chromatography on a Superdex-200 column. AFDH preferred anhydrofructose (AF) over its natural substrate D-glucosone. The concentrations of AF and D-glucosone that yielded half of the maximum activity were 12.62 mM and 27.58 mM, respectively. Vmax was estimated to be 769 units for AF and 416 units for D-glucosone. AFDH had an optimal pH range of 5.9 to 7.0 with an optimal activity at pH 6.7. AFDH had an optimum temperature range between 34°C and 46°C with an optimum temperature at 38°C. The metal ions  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Na}^{+}$  all increased the AFDH activity, while  $\text{Zn}^{2+}$ , EDTA and DTT inhibited the enzyme.

AFDH can therefore be used for the production of Ascopyrone M from AF, the precursor for ascopyrone P. Due to the discovery of AFDH converting glucosone, AFDH can therefore also be used for the production of the antimicrobial cortalcerone.

20

## 2. Materials and Methods

### 2.1 Purification of AFDH

#### 25 2.1.1. Culture of *Anthracobia Melaloma* and induction of AFDH

The fungus *A. melaloma* (CBS 293.54) was obtained from Centraalbureau voor Schimmelcultures (CBS, Baarn, NL). *A. melaloma* was grown on potato dextrose agar (PDA) medium for 20 days at 24°C. To induce the AFDH production, the mycelium was carefully removed from the agar plates and placed at -20°C for 24 hours. The biomass of 640 g was thawed at room temperature (22-24°C). Sodium phosphate buffer (50mM, pH 7.5) containing 1 % of toluene in a volume of 380ml was added to the biomass, mixed and placed at 22°C for 3 hours and then homogenized with an

30

ultraturax for at least 15 minutes. The mixture was then incubated at 4°C for 24 hours. The mixture was then centrifuged at 10000 x g at 4°C for 30 minutes and the supernatant was filtered through a Whatman filter paper. A total volume of 332 ml was obtained.

5

#### 2.1.2. Ammonium sulphate precipitation

Ammonium sulphate was added slowly to the supernatant to 40% saturation at 0°C and after 30 min at 0°C, the solution was centrifuged at 10000 x g for 30 min. To the supernatant ammonium sulphate was added to 80% saturation. After 30 minutes at 0°C, 10 the solution was centrifuged at 10000 rpm for 30 min. The supernatant was carefully removed and the pellet was resuspended in 30 ml 50 mM Na-phosphate buffer (pH 7.5). AFDH was found in the fraction precipitated from 40 to 80 % ammonium sulphate saturation. By this step a purification factor of around 11 was achieved with an extraordinary high recovery of 549% (Table 1 below), indicating the removal of 15 inhibitor(s) by this step.

#### 2.1.3. Hydrophobic interaction chromatography (HIC) separating AFDH from Ascopyrone P synthase 1 and 2

The resuspended pellet was diluted to 300 ml using 50 mM Na-phosphate buffer (pH 20 7.5) (hereafter called buffer B1) and ammonium sulphate was added to a concentration of 1.2 M. A HiLoad Phenyl Sepharose 16/10 HP column (Pharmacia) was equilibrated with 50 mM Na-phosphate buffer (pH 7.5) containing 1.2 M ammonium sulphate (hereafter called buffer A1) and the solution was applied to the column. The column was washed with buffer A1 and eluted with a stepwise gradient: linear gradient from 0- 25 55 % buffer B1 in 10 column volumes, followed by 55% buffer B1 for 5 column volumes, and then from 55 % to 100 % buffer B1 linearly in 10 column volumes (Fig 1). The flow rate was 2 ml/min. Fraction size was 3 ml.

As it can be seen from Fig. 1, AFDH was efficiently separated from ascopyrone P 30 synthase 1 (APS1) and ascopyrone synthase 2 (APS2) by this step. AFDH was first eluted with 39% buffer B1, While APS1 was eluted at 55% buffer B1 and APS2 at 100% buffer B1. A purification factor of 1.7 was achieved by this step (Table 1 below).

#### 2.1.4. Ion exchange chromatography.

1<sup>st</sup> IEC: The active fractions of AFDH were pooled (14 ml) and concentrated with centriprep 10 with a molecule cutoff of 10000 (Millipore incorporation, USA). The sample was desalted on a PD 10 gel filtration column (Pharmacia) and the buffer was changed to 20 mM Bis-Tris-Propane-HCl buffer (pH 7.5) (buffer A2), applied to a 6 ml resource Q anion exchanger column (Pharmacia) pre-equilibrated with buffer A2. After loading of the sample, the column was washed with buffer A2. AFDH was eluted with a stepwise gradient of 20 mM Bis-Tris-Propane-HCl buffer (pH 7.5) containing 1.0 M NaCl (buffer B2): linear gradient from 0-36% buffer B2 in 36 column volumes, followed by 36% buffer B2 for 20 column volumes, and then from 36% to 100% buffer B2 in 6 column volumes (Fig 2). The flow rate was 6 ml/min and the fraction size was 3 ml. AFDH was eluted at 30% Buffer B2.

2<sup>nd</sup> IEC: The active fractions was pooled and concentrated (4.5 ml) with centriprep 10. The sample was desalted on a PD-10 gel filtration column (Pharmacia) and the buffer was changed to a 20 mM Bis-Tris-Propane-HCl buffer (pH 6.0) (buffer A3), applied to a Mono Q 5/5 column (Pharmacia) pre-equilibrated with buffer A3. After loading of the sample, the column was washed with buffer A3. The elution profiles are: 0-40% buffer B3 (Buffer A3 containing 1 M NaCl) in 80 column volumes, 40-100% buffer B3 in 4 column volumes. Flow was 0.5 ml/min and fraction size was 0.5 ml. AFDH activity was found in tube 138- 152 with an elution volume of 93-96ml at 30% of buffer B3 (Fig 3). A purification factor of 18 was achieved with these two steps of ion exchange chromatography (Table 1 below).

#### 2.1.5. Gel filtration chromatography

The pooled and concentrated active AFDH fractions from the MonoQ step were loaded onto a Superdex 200 gel filtration column (Pharmacia). The column was pre-equilibrated and eluted with 50 mM Na-Phosphate buffer (pH 7.0) containing 0.1 M NaCl. The AFDH peak, (fraction 13-15), was pooled and concentrated (0.5 ml) (Fig 4). The molecular mass of AFDH was estimated to be 228 kDa from its distribution coefficient, relative to the marker proteins in kDa (ribonuclease A (13.7), ovalbumin (43), albumin (67) and aldolase (158, catalase, 232, ferritin 440). The void volume was

determined using blue dextran.

### 3. Characterisation of AFDH

- 5 The purification of AFDH procedure was followed by SDS-PAGE, and native-PAGE using PhastSystem (Pharmacia) using 8-25 % gradient gels according to the manufacturer's instruction. Visualisation of protein bands on the gels was made with Coomassie brilliant blue staining. Amino acid sequencing of AFDH was performed as described earlier (Yu *et al.*, 1997). AFDH was first partially hydrolysed with proteinases.
- 10 The generated peptide fragments were separated on HPLC. Each individual polypeptide was collected, analysed for their molecular masses by mass spectrometer, and sequenced on an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

### 4. Enzyme Assays

15

#### 4.1 Enzyme activity assay of AFDH

- The reaction mixture consisted of 50  $\mu$ l 1.5-anhydro-D-fructose (30 mg/ml), 1 to 50  $\mu$ l anhydrofructose dehydratase (AFDH) sample, 0.5 ml 50 mM Na-phosphate buffer (pH 7.5) containing 1.0 M NaCl and deionized water to a total volume of 0.7 ml. The
- 20 reaction mixture was vortexed and incubated at 22°C for 30 minutes. At the end of incubation the reaction mixture was scanned between 400-200 nm and the peak absorbance at 263 nm was recorded on a Perkin Elmer Lambda 18 uv/vis spectrophotometer. One activity unit is defined as the enzyme needed to cause an increase of 0.01 absorbance unit at 263 nm at 22°C per min.

25

#### 4.2 Preparation of AFDH product

- The product of AFDH was prepared in the same way as for the activity assay of AFDH except that more AF (final AF concentration 2-4%) was used and the reaction was performed in a membrane-reactor with a molecule cut-off of 10,000. The reaction was
- 30 followed by the increase at 263 nm. At the end of the reaction the AFDH product formed was separated from the AFDH and used for the assay of APS.

#### 4.3 Enzyme activity assay of APS:

##### a) Coupled reaction assay method with AFDH as the tool enzyme

The reaction mixture consisted of 50  $\mu$ l AF (30 mg ml<sup>-1</sup>), 1  $\mu$ l of AFDH, 0.5 ml 50 mM sodium phosphate buffer (pH 7.5) containing 1.0 M NaCl, and 149  $\mu$ l deionized water and to a total volume of 0.7 ml. The reaction mixture was vortexed and incubated at 22 °C for 30 min. At the end of incubation the reaction mixture was heated at 95°C for 1min. After cool to 22 °C, 10  $\mu$ l sample of APS was added, mixed and incubated at 22 °C for 30 min. At the end of incubation the reaction mixture was scanned between 400-200 nm and the peak absorbance at 289 nm was recorded on a Perkin Elmer Lambda 18 uv/vis spectrophotometer.

##### b) The direct assay method

The same as the coupled reaction assay method except AF and AFDH were replaced with the product of AFDH prepared from the enzyme reactor. One activity unit of APS is defined an increase of 0.01 absorbance unit at 289 nm at 22 °C per min.

The assay methods for ADH and APS were also adapted to use a microplate and microplate reader. In this case the reaction volume for AFDH and APS was reduced to 0.2 ml. At the end of the reaction 10  $\mu$ l 1 N NaOH was added to each well of the microplate to stop the reaction and the APP content was measured at 340 nm using a microplate reader (Model EAR 340 AT, SLT-Labinstruments, Grödig, Austria). For the assay of AFDH, the reaction mixture contained also APS as a tool enzyme. This method is used for fast screening the activities of AFDH and APS, such identifying the activity fractions in the chromatography steps.

25

4.4 HPLC quantification of the products of AFDH and APS: The formed product was also separated and quantified on a Waters HPLC instrument (model WISP 710B) equipped with a differential refractometer (model 410) and a uv monitor (Lambda-Max model 481 LC spectrophotometer) set at 263nm for the product of AFDH and 289nm for the product of APS. The column used was a carbohydrate Ca<sup>2+</sup> column (6.5x300mm, Interaction Chromatography Inc. San Jose, CA) and a Symmetry Shield 3.9x150mm C18 column (Waters Corporation). The structure of APP was confirmed

30

using NMR as described earlier [WO 00/56838].

## 5. Results

### 5.1 Purification and chromatography properties of AFDH

#### 5.1.1. Ammonium sulfate fractionation:

In the ammonium sulfate fractionation step, AFDH was found in the fraction precipitated from 40 to 80% ammonium sulfate saturation. By this step a purification factor of 8 was achieved with satisfactory recovery (Table 1).

#### 5.1.2. Hydrophobic interaction chromatography

AFDH was further purified and efficiently separated from APS by hydrophobic interaction chromatography on HiLoad Phenyl Sepharose 16/10 HP. AFDH was first eluted with 39 % Buffer B, while APS1 at 55% Buffer B, APS2 at 100% Buffer B (Figure 1).

#### 5.1.3. Ion exchange chromatography

The desalted fractions of AFDH were further purified on an anion exchanger Mono Q 5/5 column AFDH was eluted at 30% Buffer B1.

#### 5.1.4. Gel filtration chromatography

The AFDH reaction from the ion exchanger step was further separated two times on a gel filtration column Superdex 200. AFDH showed one single symmetric peak. A molecular mass of 170 kDa was estimated from its elution volume.

#### 5.1.5. SDS-PAGE analysis

The separated three fractions of AFDH from the ion exchange step were analysed by SDS-PAGE. All the three fractions showed one single band with a relative molecular mass ( $M_r$ ) of 97kDa.



**Table 1.** A summary of the purification steps for AFDH from *Anthracobia melaloma*.

Fraction	ml	Protein mg/ml	Total protein mg	activity units/ml	Total activity	Specific activity	Purification fold	Yield %
Cell-free extract	332.0	0.135	44.80	376.0	124832.0	2786.4	1	100
Ammonium sulfate precipitation	30.0	0.730	21.90	22843.3	685300.0	31292.2	11	549
HIC	14.0	0.680	9.50	35910.0	502740.0	52920.0	19	403
IEC (Resource Q)	4.5	0.410	1.85	96506.7	434280.2	234746.0	84	348
IEC (Mono Q)	0.8	0.350	0.28	335797.0	268637.6	959420.2	344	215
Gel filtration	0.5	0.430	0.22	501815.0	250907.5	1140488.7	409	201

From Table 1 it can be seen that AFDH has been purified by a factor of 409 with a high recovery of 201%. The finally purified AFDH was examined on SDS-PAGE using PhastSystem (Pharmacia) and pre-cast gels with a gel gradient of 8-25% according to the manufacturer's instructions. AFDH showed a single band with a molecular mass of 98.5 kDa (Fig. 5).

## 5.2 Characterisation of AFDH

### 5.2.1 The optimum temperature of AFDH

The reaction mixture consisted of 500  $\mu$ l 50 mM Mes-NaOH (pH 6.7), 200  $\mu$ l AF (0.185 mmol/ml), and deionised water to a total volume of 1.4 ml. 1  $\mu$ l of AFDH (502 units) was added to the reaction mixture. The reaction mixture was vortexed and incubated 15 min at different temperatures (4°C-60°C).

**Table 2.** AFDH activity as a function of temperature

Temperature C°	OD263	Activity units	Temperature C°	OD263	Activity units
4	0.291	2716.00	42	0.838	7821.33
10	0.374	3490.67	44	0.781	7289.33
24	0.527	4918.67	46	0.744	6944.00
28	0.596	5562.67	50	0.621	5796.00
30	0.690	6440.00	52	0.529	4937.33
34	0.779	7270.67	54	0.486	4536.00
36	0.814	7597.33	56	0.375	3500.00
38	0.869	8110.67	60	0.277	2585.33
40	0.851	7942.67			

From Table 2, it can be seen that AFDH had an optimum temperature range from 34°C to 50°C with an optimum temperature at 38°C. At temperature above 50°C the activity of AFDH decreased rapidly.

### 5.2.2 pH optimum of AFDH

The reaction mixture consisted of 500 µl 0.1 M buffer HAc-NaAc (pH 4.1-5.5), Mes-NaOH (pH 5.5-6.7), Mops-NaOH (pH 6.0-8.0) and Bicine-NaOH (pH 7.6-9.0), 100 µl 1.0 M NaCl, 200 µl substrate AF (0.185 µmol/ml), 1 µl of AFDH and deionised water to a total volume of 1 ml. The reaction mixture was vortexed and incubated at 22°C for 30 min (Table 3).

**Table 3.** The effect of pH on the activity of AFDH.

Buffer	pH	OD263	Activity units	Buffer	PH	OD263	Activity units
HAc-NaAc	4.1	0.058	193.33	Mops-NaOH	6.5	1.209	4030.00
HAc-NaAc	4.5	0.065	216.67	Mops-NaOH	7.0	0.993	3310.00
HAc-NaAc	5.0	0.087	290.00	Mops-NaOH	7.5	0.652	2173.33
HAc-NaAc	5.5	0.257	856.67	Mops-NaOH	8.0	0.500	1666.67
Mes-NaOH	5.5	0.392	1306.67	Bicine-NaOH	7.6	0.705	2350.00
Mes-NaOH	5.9	0.872	2906.67	Bicine-NaOH	7.9	0.639	2130.00
Mes-NaOH	6.3	1.238	4126.67	Bicine-NaOH	8.2	0.608	2026.67
Mes-NaOH	6.7	1.332	4440.00	Bicine-NaOH	8.6	0.536	1786.67
Mops-NaOH	6.0	1.070	3566.67	Bicine-NaOH	9.0	0.487	1623.33

From Table 3, one can see that AFDH from *A. melaloma* had an optimal pH range of 5.9 to 7.0 with optimal activity at pH 6.7. The enzyme activity decreased dramatically in pH values lower than 5.9 and a decrease in activity was also observed in pH values higher than 7.0.

5

### 5.2.3 Effectors

The influence of the following ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{Zn}^{2+}$ ), EDTA and DTT on the activity of AFDH was examined. The reaction mixture consisted of 250  $\mu\text{l}$  0.1 M Mes-NaOH (pH 6.7), 205  $\mu\text{l}$  AF (127.9  $\mu\text{mol/ml}$ ), 0-50 mM  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  or  $\text{ZnCl}_2$ ,  
 10 or 0-0.9 M NaCl, or EDTA 10, 50 mM or 10 mM DTT and deionised water to a total volume of 1 ml and 2  $\mu\text{l}$  AFDH was added. The reaction mixture contained 502 U of enzyme. The reaction mixture was vortexed and incubated 30 minutes at 22°C.

Table 4.  $\text{CaCl}_2$  and  $\text{MgCl}_2$  effect on AFDH activity

$\text{CaCl}_2$ mM	OD263	Activity units	$\text{MgCl}_2$ mM	OD263	Activity units
0	0.396	660.0	0	0.391	651.7
5	0.747	1245.0	5	0.632	1053.3
10	0.865	1442.7	10	0.737	1228.3
15	0.873	1455.0	15	0.753	1255.0
20	0.919	1531.7	20	0.777	1295.0
25	0.937	1561.7	25	0.790	1316.7
30	0.961	1601.7	30	0.775	1291.7
40	0.884	1473.3	40	0.746	1243.3
50	0.880	1466.7	50	0.712	1186.7

15

65

**Table 5. NaCl and ZnCl<sub>2</sub> effect on AFDH activity**

NaCl M	OD263	Activity units	ZnCl <sub>2</sub> mM	OD263	Activity units
0	0.341	568.3	0	0.497	828.3
0.05	0.758	1263.3	5	0.331	551.7
0.1	0.809	1348.3	10	0.261	435.0
0.15	0.829	1381.7	15	0.189	315.0
0.2	0.849	1415.0	20	0.145	241.7
0.3	0.872	1453.3	25	0.132	220.0
0.4	0.803	1338.3	30	0.121	201.7
0.5	0.781	1301.7	40	0.095	158.3
0.6	0.772	1286.7	50	0.081	135.0
0.7	0.763	1271.7			
0.8	0.734	1223.3			
0.9	0.685	1141.7			

**Table 6. Inhibition of AFDH**

Inhibitor	Concn (mM)	Relative activity (%)
None		100
DTT	10	57
EDTA	10	59
EDTA	50	16
Zn <sup>2+</sup>	10	52.52

5

From Table 4, it can be seen that 25 mM MgCl<sub>2</sub> or 10 mM CaCl<sub>2</sub> was found to increase the activity with a factor of two. Na<sup>+</sup> also had also a positive effect on AFDH activity and a concentration of 50 mM NaCl was needed to achieve the same enhancement as observed for MgCl<sub>2</sub> and CaCl<sub>2</sub>. On the other hand ZnCl<sub>2</sub>, EDTA and DTT inhibited AFDH (Tables 5-6).

10

AFDH was stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two week at 4°C.

15

#### 5.2.4 The effect of substrate concentration on activity

The activity of AFDH was measured as a function of the 1.5-anhydro-D-fructose and D-glucosone concentration. The reaction mixture consisted of 500  $\mu$ l 0.1 M Mes-NaOH buffer (pH 6.7) containing 1.0 M NaCl, 5-480  $\mu$ l substrate AF (127.9  $\mu$ mol/ml) or 5-300  $\mu$ l D-glucosone (247.45  $\mu$ mol/ml) and deionised water to a total volume of 1.0 ml and 5  $\mu$ l of AFDH was added to the mixture. The reaction mixture contained 502 U of enzyme. The reaction mixture was vortexed and incubated at 22°C for 30 min. The activity of AFDH incubated with AF was measured as described above. The formation of cortalcerone from D-glucosone was measured at 265 nm.

10

**Table 7.** AFDH activity as a function of AF concentrations

Substrate $\mu$ l	Substrate ( $\mu$ mol/ml)	OD263	Activity (units)	Substrate $\mu$ l	Substrate ( $\mu$ mol/ml)	OD263	Activity (units)
5	0.64	0.059	39.33	160	20.46	0.767	511.33
10	1.28	0.094	62.67	180	23.02	0.824	549.33
20	2.56	0.152	101.33	200	25.58	0.842	561.33
30	3.84	0.274	182.67	230	29.42	0.892	594.67
40	5.12	0.316	210.67	260	33.25	0.914	609.33
50	6.40	0.328	218.67	300	38.37	0.97	646.67
60	0.46	0.384	256.00	400	51.16	1.108	738.67
70	8.95	0.491	327.33	450	57.56	1.151	767.33
100	12.79	0.618	412.00	480	61.39	1.159	772.67
130	16.63	0.685	456.67				

**Table 8.** AFDH activity as a function of D-glucosone concentrations.

Substrate $\mu$ l	Substrate $\mu$ mol/ml	OD265	Activity units	Substrate $\mu$ l	Substrate $\mu$ mol/ml	OD265	Activity units
5	1.24	0.050	33.33	90	22.27	0.323	215.30
10	2.47	0.054	36.00	100	24.75	0.346	230.67
20	4.95	0.086	57.33	120	29.69	0.379	262.67
30	7.42	0.119	79.30	150	37.12	0.394	310.20
40	9.90	0.152	101.33	170	42.07	0.529	352.62
50	12.37	0.171	114.00	200	49.49	0.592	394.62
60	14.85	0.211	140.67	250	61.86	0.611	407.33
70	17.32	0.247	164.67	300	74.24	0.622	414.67
80	19.80	0.271	180.67				

Using the data from Tables 7 and 8, the apparent  $K_m$  and  $V_{max}$  were estimated to 12.62 mM and 769 units for AF; 27.58 mM and 416 units for D-glucosone.

### 5 5.3 Antibody production

Antibodies were raised against the amino acid of the present invention by injecting rabbits with the purified enzyme and isolating the immunoglobulins from antiserum according to procedures described according to N Harboe and A Ingild ("Immunization, Isolation of  
10 Immunoglobulins, Estimation of Antibody Titre" In A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, N H Axelsen, *et al.* (eds.), Universitetsforlaget, Oslo, 1973) and by T G Cooper ("The Tools of Biochemistry", John Wiley & Sons, New York, 1977).

15 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed  
20 should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

**CLAIMS**

1. 1,5-Anhydro-D-fructose dehydratase in isolated or purified form.
2. 1,5-Anhydro-D-fructose dehydratase according to claim 1 which has a temperature optimum of from about 34 to 50 °C.
3. 1,5-Anhydro-D-fructose dehydratase according to claim 1 or claim 2 which has a temperature optimum of about 38 °C.
4. 1,5-Anhydro-D-fructose dehydratase according to any one of claims 1 to 3 which has an optimal pH range of from about 5.9 to about 7.0.
5. 1,5-Anhydro-D-fructose dehydratase according to any one of claims 1 to 4 which has an optimal pH of about 6.7.
6. 1,5-Anhydro-D-fructose dehydratase according to any one of claims 1 to 5 which is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.
7. 1,5-Anhydro-D-fructose dehydratase according to any one of claims 1 to 6 which has the following characteristics:
  - (i) a temperature optimum of from about 34 to about 50 °C;
  - (ii) an optimal pH range of from about 5.9 to about 7.0; and
  - (iii) is stable in 50mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least two weeks at 4°C.
8. 1,5-Anhydro-D-fructose dehydratase according to any preceding claim which is sensitive to ZnCl<sub>2</sub>, EDTA or DTT.
9. 1,5-Anhydro-D-fructose dehydratase according claim 8 which is inhibited by ZnCl<sub>2</sub>, EDTA or DTT.

10. 1,5-Anhydro-D-fructose dehydratase according to any preceding claim which is sensitive to  $Mg^{2+}$ ,  $Ca^{2+}$  or  $Na^{+}$  ions.
11. 1,5-Anhydro-D-fructose dehydratase according to any preceding claim which exhibits enhanced activity in the presence of  $Mg^{2+}$ ,  $Ca^{2+}$  or  $Na^{+}$  ions.
12. A process for preparing ascopyrone P using 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11.
13. A process according to claim 12 wherein said process further comprises the use of ascopyrone P synthase.
14. A process according to claim 13 which comprises contacting ascopyrone P synthase and 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11 with 1,5-anhydro-D-fructose.
15. A process according to claim 13 or claim 14 which further comprises the use of  $\alpha$ -1,4-glucan lyase.
16. A process according to claim 15 comprising contacting  $\alpha$ -1,4-glucan lyase, ascopyrone P synthase and 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11 with a starch-type substrate.
17. A process according to claim 16 which comprises the steps of:
  - (i) contacting  $\alpha$ -1,4-glucan lyase with a starch-type substrate;
  - (ii) contacting the product from step (i) with ascopyrone P synthase 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11.
18. A process according to claim 16 or claim 17 wherein said starch-type substrate is selected from glycogen or a maltodextrin.
19. A process for preparing ascopyrone M using 1,5-anhydro-D-fructose dehydratase



according to any one of claims 1 to 11.

20. A process according to claim 19 which comprises contacting 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11 with 1,5-anhydro-D-fructose.

21. A process for preparing cortalcerone using 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11.

22. A process according to claim 21 which comprises contacting 1,5-anhydro-D-fructose dehydratase according to any one of claims 1 to 11 with glucosone.

23. An enzyme having 1,5-anhydro-D-fructose dehydratase activity substantially as described herein and with reference to the accompanying Examples.

24. A process for preparing ascopyrone P substantially as described herein and with reference to the accompanying Examples.

25. A process for preparing ascopyrone M substantially as described herein and with reference to the accompanying Examples.

26. A process for preparing cortalcerone P substantially as described herein and with reference to the accompanying Examples.

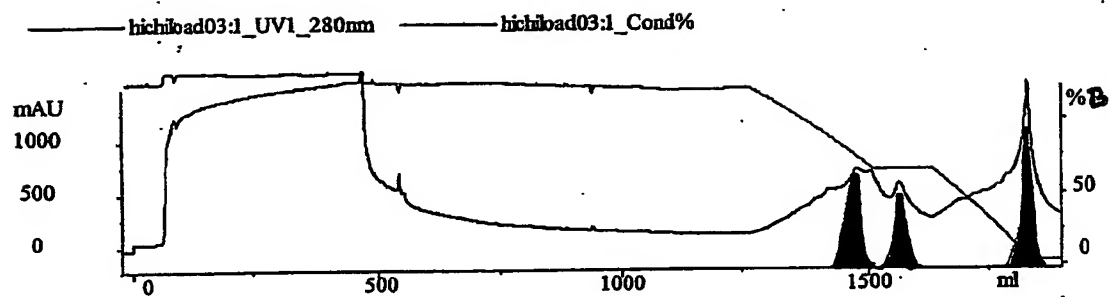


FIGURE 1

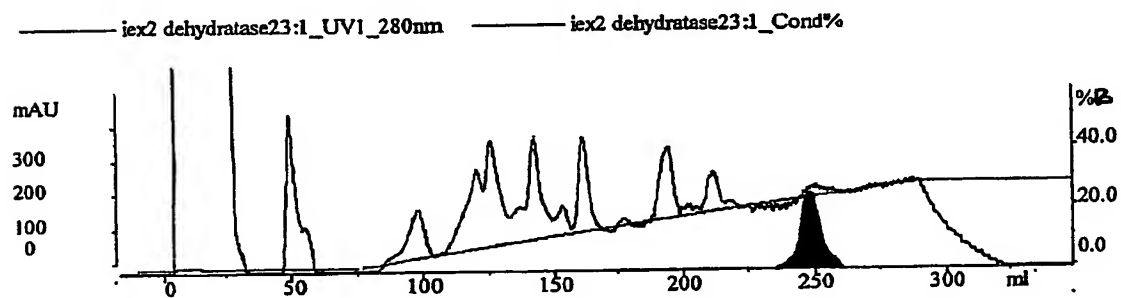


FIGURE 2

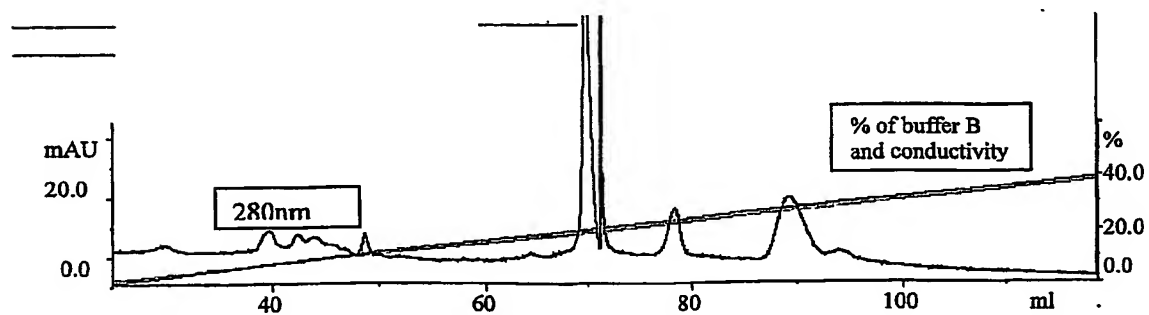


FIGURE 3

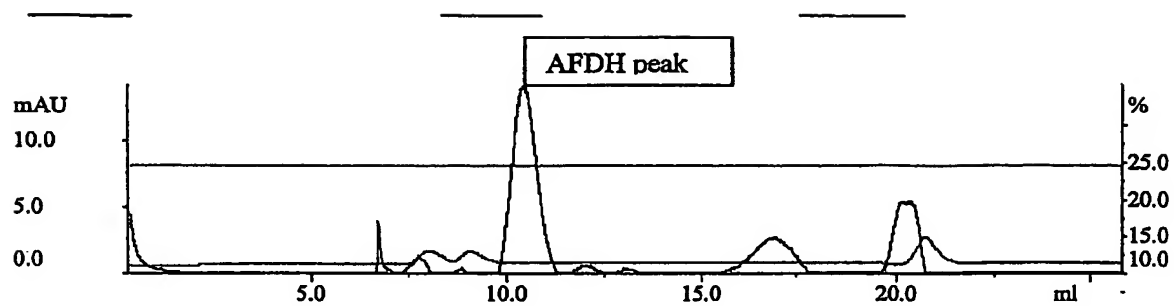


FIGURE 4

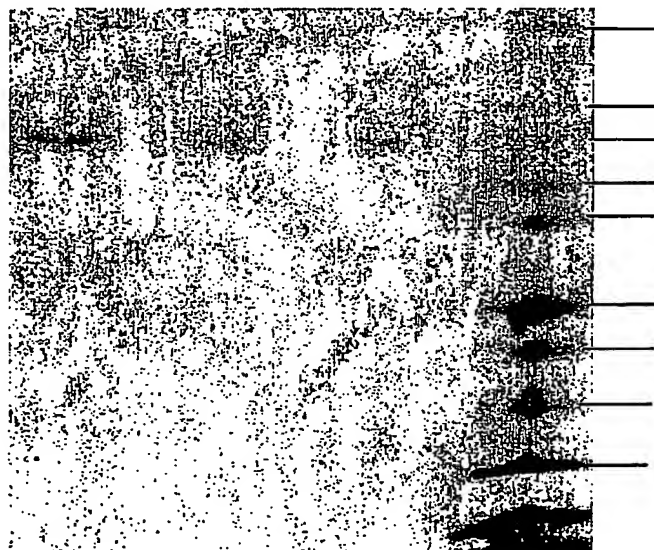


FIGURE 5

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 02/04951

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N9/88 C07D309/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, BIOSIS, EPO-Internal, WPI Data, PAJ, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 00 56838 A (ANDERSEN SOEREN MOELLER ; JENSEN HENRIK MAX (DK); DANISCO (DK); ; EA) 28 September 2000 (2000-09-28) page 9, line 4-12 page 10, line 1-3	1-20
	BAUTE M-A ET AL: "ENZYME ACTIVITY DEGRADING 1,4-ALPHA-D-GLUCANS TO ASCOPYRONES P AND T IN PEZIZALES AND TUBERALES" PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 33, no. 1, 1993, pages 41-45, XP000925242 ISSN: 0031-9422 cited in the application the whole document -/--	1-18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*G\* document member of the same patent family

Date of the actual completion of the international search

5 February 2003

Date of mailing of the international search report

28/02/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Herrmann, K

## INTERNATIONAL SEARCH REPORT

 Int. Application No  
 PCT/GB 02/04951

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAUTE R ET AL: "PROPOSED PATHWAY TO THE PYRONES CORTALCERONE AND MICROTHECIN IN FUNGI" PHYTOCHEMISTRY (OXFORD), vol. 26, no. 5, 1987, pages 1395-1398, XP009005230 ISSN: 0031-9422 the whole document, especially Experimental	1-11, 21, 22
X	DEFFIEUX G ET AL: "1,5-D-ANHYDROFRUCTOSE, THE PRECURSOR OF THE PYRONE MICROTHECIN IN MORCHELLA VULGARIS" PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 26, no. 5, 1987, pages 1391-1393, XP000999128 ISSN: 0031-9422 page 1393, left-hand column	1-11
A	KOTHS K ET AL: "SYNTHESIS OF THE ANTIBIOTIC CORTALCERONE FROM D GLUCOSE USING PYRANOSE 2-OXIDASE AND A NOVEL FUNGAL ENZYME ALDOS-2-ULOSE DEHYDRATASE" CARBOHYDRATE RESEARCH, vol. 232, no. 1, 1992, pages 59-75, XP009005251 ISSN: 0008-6215 the whole document	21, 22
A	BOJSEN KIRSTEN ET AL: "A group of alpha-1,4-glucan lyase genes from the fungi Morchella costata, M. vulgaris and Peziza ostracoderma. Cloning, complete sequencing and heterologous expression." PLANT MOLECULAR BIOLOGY, vol. 40, no. 3, June 1999 (1999-06), pages 445-454, XP002230018 ISSN: 0167-4412 cited in the application the whole document	1-22
P, X	WO 02 26060 A (ELSSER DIETER ; DANISCO (DK); MORGAN ANDREW JOHN (DK); THOMAS LINDA) 4 April 2002 (2002-04-04) page 9, line 13 - line 21 page 10, line 8 - line 11	1-20

-/-

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 02/04951

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THOMAS L V ET AL: "Ascopyrone P, a novel antibacterial derived from fungi." JOURNAL OF APPLIED MICROBIOLOGY, vol. 93, no. 4, 2002, pages 697-705, XP002230019 2002 ISSN: 1364-5072 page 697, right-hand column, line 1-3 page 701, right-hand column, last paragraph</p>	1-22

## INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 23-26

Due to the wording "substantially as described herein and with reference to the accompanying examples" claims 23-26 are not allowable under Rule 6.2(a) PCT and fail to comply with the requirements of Art. 6 PCT (clarity) to such an extent that a meaningful search could not be carried out (Art. 17(2)(a)(ii) PCT).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 02/04951

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(e) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 23-26  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Int. Patent Application No  
PCT/GB 02/04951

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0056838	A	28-09-2000	AU	3315800 A	09-10-2000
			BR	0008689 A	08-01-2002
			CA	2362265 A1	28-09-2000
			CN	1344306 T	10-04-2002
			EP	1169409 A1	09-01-2002
			WO	0056838 A1	28-09-2000
			JP	2002540248 T	26-11-2002
			US	2002051840 A1	02-05-2002
WO 0226060	A	04-04-2002	AU	9013301 A	08-04-2002
			AU	9013501 A	08-04-2002
			WO	0226060 A1	04-04-2002
			WO	0226061 A1	04-04-2002

**THIS PAGE BLANK (USPTO)**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ ~~SKewed/SLANTED IMAGES~~
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**